A novel phospholipase A_2 from human placenta

Wolf-Juergen BUHL,* Liisa M. EISENLOHR, Ilka PREUSS and Ulrich GEHRING
Institut für Biologische Chemie, Universität Heidelberg, Im Neuenheimer Feld 501, D-69120 Heidelberg, Germany

A major soluble phospholipase A_2 of human term placenta was characterized and purified about 15000-fold to homogeneity. The apparent molecular mass as determined in SDS/polyacrylamide gels is 42 kDa. The enzyme is inhibited by dithiothreitol indicating the presence of disulphide bridges which are essential for activity. Studies with known phospholipase A_2 inhibitors revealed no immediate relationship to either secretory or cytosolic phospholipases A_2. The placental enzyme prefers liposomes of phosphatidylcholine and has a distinct preference for arachidonic acid in the *sn*-2 position. It tolerates various detergents. Roughly 10 μM Ca^{2+} is required for activity, but it cannot be replaced by Mg^{2+} or Mn^{2+}; Zn^{2+}, Cu^{2+} and Fe^{3+} are inhibitory. In immunoblots, the placental enzyme was not detected by two separate antisera specific for type-II phospholipases A_2 but reacted very weakly with antisera directed against cytosolic phospholipase A_2. From these data we suggest that this enzyme is a novel form of phospholipase A_2 which may be involved in arachidonic acid mobilization both during the course of pregnancy and at parturition.

INTRODUCTION

The biosynthesis of eicosanoids in human placenta is of particular interest in view of their key regulatory role during pregnancy. Eicosanoids have been implicated both in the regulation of placental vessel function [1–3] and, later at term, in the initiation of labour and parturition [4–7]. Depending on the type produced, eicosanoids elicit opposing physiological effects on smooth-muscle activity, some being vasodilatory and others acting to constrict vessels [1,3]. Decreased production of vasodilator eicosanoids has been observed in situations of restricted uteroplacental blood flow [1,3]. This may be causally related to pregnancy-induced hypertensive diseases, most notably pre-eclampsic toxaemia, a serious complication which in many cases is lethal to child and mother [1,2,8]. On the contrary, just before parturition, the production of vasoconstrictor eicosanoids not only dramatically increases in gestational tissues, but also the pattern changes to compounds that stimulate uterine contractility [4]. Dysregulated production of these types of eicosanoids has been suggested to lead to preterm delivery, one of the leading causes of perinatal morbidity and mortality [4–7].

All members of the eicosanoid family of lipid molecules are metabolically derived from arachidonic acid. As most cellular arachidonate is stored in an esterified form in the *sn*-2 position of glycerophospholipids, the release from such phospholipids largely determines eicosanoid biosynthesis. Phospholipases A_2 (PLA_2s) are of major importance as they specifically hydrolyse the 2-acyl ester bond of phospholipids thus mobilizing arachidonic acid in a one-step reaction [9–12]. However, alternative methods of arachidonate release also operate in various cell types, but require more than one enzyme [13–15].

A wealth of enzymes with PLA_2 activity has been described. The majority of them are members of two distinct gene families: the exclusively cytosolic PLA_2 ($cPLA_2$) of molecular mass 85 kDa and secretory PLA_2s of molecular mass about 14 kDa ($sPLA_2$s), the latter comprising a variety of members. Soluble PLA_2s may be either cell-associated or secreted. This class includes type-I PLA_2, originally detected in pancreatic exudate, and type-II PLA_2s, found in both cells and extracellular fluids under inflammatory conditions [9–12]. $cPLA_2$ is known for its specificity towards arachidonyl phospholipids [9,10,16]. This enzyme has evoked great interest during recent years as cellular stimulation can lead to its phosphorylation and activation in response to elevated intracellular Ca^{2+} levels [10,17,18]. Recently, it has been shown to be involved in arachidonic acid release from stimulated platelets [19,20]. However, there is also evidence for involvement of type-II and other PLA_2s in arachidonic acid mobilization under various cell stimulatory conditions [11,21–24].

We became interested in the PLA_2 forms in human term placenta and reported on several soluble activities of different ionic properties and substrate specificities [25]. In the present investigation we biochemically characterized the major PLA_2 activity of this tissue and purified it to homogeneity. We find that this enzyme is a novel form of PLA_2 distinct from both type-II PLA_2 and $cPLA_2$.

MATERIALS AND METHODS

Materials

1-Palmitoyl-2-[1-^{14}C]palmitoylphosphatidylcholine (2.29 GBq/mmol) and 1-stearoyl-2-[1-^{14}C]arachidonylphosphatidylcholine (2.07 GBq/mmol) were purchased from Amersham Buchler (Braunschweig, Germany). *p*-Bromophenacyl bromide and *n*-octyl-D-glucoside were from Fluka (Buchs, Switzerland). PLA_2 from *Trimeresurus flavoviridis* venom was obtained from Calbiochem-Novabiochem (Bad Soden, Germany). All other biochemicals used were of analytical grade and were purchased from Sigma (München, Germany). Blue Dextran 2000, DEAE-Sephadex A-50, CM-Sephadex C-50 and phenyl-Sepharose CL-4B were from Pharmacia Biotech (Freiburg, Germany). Bio-Gel HT hydroxyapatite was obtained from Bio-Rad (München, Germany), and Ultrogel AcA 44 was from Serva (Heidelberg, Germany).

Abbreviations used: DTT, dithiothreitol; PLA_2, phospholipase A_2 (EC 3.1.1.4); $cPLA_2$, cytosolic PLA_2; $sPLA_2$, secretory PLA_2.

* To whom correspondence should be addressed.

PLA_2 assay

The standard assay mixture (100 μl) contained 6 μg of liposomes in 20 mM Hepes buffer, pH 7.5, containing 150 mM NaCl and 1 mM $CaCl_2$. The reaction was started by the addition of the fraction to be tested and incubation was at 37 °C routinely for 60 min. Liposomes were prepared as described previously [25]. For routine assays, we used liposomes of dipalmitoylphosphatidylcholine containing 20000 c.p.m. of 1-palmitoyl-2-[^{14}C]palmitoylphosphatidylcholine per assay. In several experiments, equivalent liposomes with 1-stearoyl-2-[1-^{14}C]arachidonylphosphatidylcholine were used instead. To check for fatty acid preference, either of the radiolabelled phosphatidylcholine phospholipids was used individually, but in dispersed rather than in liposome form [17,26]. Reactions were stopped by dilution into 2 ml of Dole reagent (propan-2-ol/heptane/0.5 M H_2SO_4, 400:100:20, by vol) and lipid was extracted as described [25]. Uncleaved phospholipid was removed on silicic acid [27] and the radiolabelled non-esterified fatty acids were quantified by liquid-scintillation counting.

PLA_2 purification from human placenta

Non-pathological placentae obtained from spontaneous deliveries at term were immediately placed on ice and further processed as detailed elsewhere [28]. Washed tissue pieces were kept frozen at −70 °C until they were thawed and minced in 10 mM Hepes buffer, pH 7.5, in the presence of protease inhibitors [25]. Routinely, we used two to three placentae per preparation. The homogenate was spun free of debris, cell organelles and low-molecular-mass particles to obtain the 100000 *g* supernatant [25].

DEAE-Sephadex and CM-Sephadex effluent

For the separation of the soluble PLA_2 forms of the 100000 *g* supernatant we used DEAE-Sephadex and CM-Sephadex [25], but by the batch technique rather than by chromatography. This combined procedure is known to remove both type-II PLA_2 ([29]; W.-J. Buhl, L. M. Eisenlohr, I. Preuss and U. Gehring, unpublished work) and $cPLA_2$ [16,30]. Activity not adsorbed to either ion exchanger (previously called S2) [25] was concentrated 20-fold in an Amicon ultrafiltration cell fitted with a PM-10 membrane. Denatured material was removed by brief ultracentrifugation (30 min at 100 000 *g*).

Phenyl-Sepharose chromatography

The concentrated sample (about 4000 mg of protein) was adjusted to 1 M NaCl and then chromatographed on a column (19 cm × 5 cm) of phenyl-Sepharose pre-equilibrated with 10 mM Hepes buffer (pH 7.5)/1 M NaCl. Protein was monitored by continuous measurement of A_{280}. Elution was at a flow rate of 1 ml/min first with 800 ml of buffer at 1 M NaCl and then with 600 ml of plain buffer. The latter wash contained less than 10% activity. For elution, 5% (w/v) octyl glucoside in Hepes was used and the resulting eluate (100–140 ml) was dialysed against 20 mM potassium phosphate buffer, pH 7.3.

Hydroxyapatite chromatography

After dialysis, the sample (about 400 mg of protein) was chromatographed on a hydroxyapatite column (24 cm × 2.6 cm) pre-equilibrated with the dialysis buffer. Chromatography was performed with 200 ml of this buffer at a flow rate of 0.5 ml/min. For elution, a linear gradient (200 ml) from 20 to 500 mM potassium phosphate (pH 7.3) was applied. Fractions with PLA_2 activity were pooled and concentrated about 60-fold by Amicon PM-10 ultrafiltration to 25–30 mg of protein/ml.

Preparative non-reducing SDS/PAGE

The concentrated fraction was made 2% (w/v) in SDS but was not treated with either 2-mercaptoethanol or dithiothreitol (DTT) and was not boiled. Some 10–15 portions were loaded on to 6 mm-wide lanes (0.4 mg of protein/lane) of a 12% (w/v) separating polyacrylamide gel (1 mm) in 0.1% (w/v) SDS with marker lanes on either side (first gel). The standards were BSA (66.5 kDa), ovalbumin (45 kDa), porcine heart lactate dehydrogenase (36.5 kDa) and bovine carbonic anhydrase (29 kDa). After electrophoresis, the gel area of molecular mass 36–47 kDa was excised, slices were crushed, and protein was eluted by shaking overnight in 10 mM Hepes. After centrifugation (10000 *g*; 5 min), the supernatant was re-electrophoresed under the same conditions (second gel); the sharply excised 42 kDa material was eluted, precipitated with 6 vol. of ice-cold acetone, air-dried, redissolved in Hepes buffer and assayed for activity.

Peptide antisera

Peptides corresponding to amino acids 6–19 (PYQHIIVEHQYSHK) and 42–58 (PDPYVELFISTTPDSRK) of human $cPLA_2$ [31] (the latter represents part of the Ca^{2+}-dependent lipid-binding domain of the enzyme) and to amino acids 10–21 (KLTTGKEAALSY) of human type-II PLA_2 [32] were synthesized on an Applied Biosystems model 430A synthesizer and coupled to haemocyanin [33]. Rabbits were immunized [33] using AdjuPrime (Pierce). Responsiveness was checked by ELISA [34] with uncoupled peptides. High titres were attained after six injections, and blood was collected 2 weeks after the last booster. Antiserum against C-terminal sequences of $cPLA_2$ [26] was provided by Dr. G. Fürstenberger (German Cancer Research Center, Heidelberg, Germany).

Analytical methods

Gel-permeation chromatography

Ultrogel AcA 44 gel filtration (column: 90 cm × 1.6 cm) was carried out at a flow rate of 0.2 ml/min using BSA, bovine carbonic anhydrase and cytochrome *c* from horse heart as standard proteins. Fractions of 1.0 ml were collected and portions thereof were assayed for PLA_2 activity. Internal solvent volume (V_i) and void volume (V_o) were determined by the use of tritiated water and Blue Dextran 2000 respectively. Distribution coefficients K_D were computed from $K_D = (V_e - V_o)/V_i$ where V_e is the elution volume of the enzyme and marker proteins. The Stokes' radius of the enzyme was obtained from plots of K_D values against the log of Stokes' radii of the markers.

SDS/PAGE and immunoblotting

Protein samples (amounts per 6 mm lane are specified in the legends to Figure 5 and Table 4) were made 2% (w/v) in SDS and were run under standard reducing conditions on 12% (w/v) separating polyacrylamide gels. Proteins were either visualized by silver staining [35] or transferred [28] to Immobilon-P membranes (Millipore). Immunostaining was with rabbit anti-peptide sera (all diluted 1:1000), with anti-$cPLA_2$ serum (raised against the human recombinant protein and kindly donated by Dr. J. L. Knopf (Genetics Institute, Cambridge, MA, U.S.A.); diluted 1:500), and with an antiserum against type-II PLA_2 from *Trimeresurus flavoviridis* venom (obtained from Dr. G. Fürstenberger; diluted 1:2500). Antibody detection was with peroxidase-

conjugated second antibody (goat anti-rabbit) and the enhanced chemiluminescence technique (Amersham).

Protein determination

Protein concentrations were determined by a microscale Bradford assay with IgG as standard [25].

RESULTS

Properties of the placental PLA_2

In this paper we have studied the major PLA_2 form of human term placenta which amounts to about half of the soluble activity [25]. This enzyme is uncharged at neutral pH and consequently does not bind to either DEAE or CM ion-exchange matrices. It was purified roughly 3-fold over the clear tissue extract with a recovery of over 100% (Table 1). This increase in activity may well be due to removal of inhibitory material as described by others [32]. We should emphasize that the enzyme at this stage of purification is devoid of other PLA_2 activities [25]; in particular, type-II and $cPLA_2$ [16,29,30] are removed by these ion-exchange chromatographic methods. Moreover, two separate antisera against type-II PLA_2 and two against $cPLA_2$ (see Table 4) were used for immunoblotting but did not produce any signals (results not shown).

The partially purified placental PLA_2 has a pH optimum between 6 and 9.5. Even though there was no turnover at pH 5, activity was regained in neutral conditions after 60 min at pH 5 (results not shown). The enzyme prefers phosphatidylcholine as substrate [25], cleavage being about 20-fold more efficient with arachidonic acid in the 2-position of the phospholipid than with palmitic acid in this position (Table 2). With mixed liposomes, containing various lipids to reflect the composition of plasma membranes [25], the enzyme showed the same preference for arachidonic acid in the 2-position (results not shown). Moreover, a similar substrate preference was also observed when dispersed phosphatidylcholine was used instead of liposomes (results not shown). In contrast, oleate-labelled *Escherichia coli* membranes [9] were barely accepted as substrate by the placental enzyme although $sPLA_2$s readily hydrolyse these membranes. This shows that the placental PLA_2 preparation is not contaminated by such activity.

Placental PLA_2 activity tolerated up to 1% Triton X-100 and Nonidet P40 and up to 0.5% sodium cholate and octyl glucoside (results not shown). At lower detergent concentrations the relative enzyme activity was even higher, suggesting that the substrate rather than the enzyme is affected [36]. Placental PLA_2 did not bind to an ATP affinity matrix and tolerated precipitation with acetone (results not shown). The enzyme is quite temperature stable with only 20% loss in activity over 15 min at 56 °C.

Gel-permeation chromatography was carried out on Utrogel AcA 44 (Figure 1). The enzyme was eluted in a symmetrical peak with a Stokes' radius of 2.6 nm (average of nine experiments), which corresponds to a molecular mass of about 30 ± 10 kDa (means $\pm$ S.D.) if one assumes a roughly globular shape.

Table 2 Substrate specificity

Enzyme purified by DEAE- and CM-Sephadex chromatography (125 mg) or to homogeneity (roughly 0.1 μg) was tested in triplicate (incubation time 10 min) with liposomes of the indicated phospholipids. The activity after SDS/PAGE is not accurate because of difficulties in measuring and handling such small amounts of protein; however, the ratio between the two activities with the substrates is reliable.

	Activity (pmol/min per mg)	
	1-Palmitoyl-2-[^{14}C]palmitoyl-phosphatidylcholine	1-Stearoyl-2-[^{14}C]arachidonyl-phosphatidylcholine
After DEAE- and CM-Sephadex	41	1080
After SDS/PAGE (first gel)	12500	242500

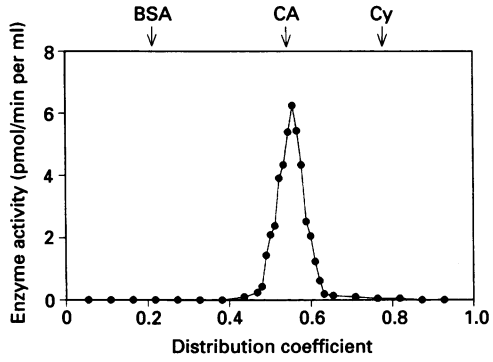

Figure 1 Gel-permeation chromatography of placental PLA_2

Enzyme purified over DEAE- and CM-Sephadex (300 μg) was submitted to gel filtration on Ultrogel AcA 44 in 10 mM Hepes buffer, pH 7.5, containing 200 mM NaCl. Marker proteins were BSA, carbonic anhydrase (CA) and cytochrome *c* (Cy) with Stokes' radii of 3.7, 2.4 and 1.7 nm respectively. The data are presented in the form of distribution coefficients.

Table 1 Purification of PLA_2

Results are for one placenta (mean of five).

	Protein (mg)	Activity		Yield (%)	Purification (fold)
		Total (nmol/min)	Specific (nmol/min per mg)		
Tissue extract (100000 *g* supernatant)	6040	75	0.012	100	= 1
DEAE- and CM-Sephadex	2050	84	0.041	112	3.4
Phenyl-Sepharose and dialysis	197	95	0.482	127	40.2
Hydroxyapatite	20.8	58	2.79	77	233
Preparative electrophoresis	0.03	5.6	186.7	7.5	15560

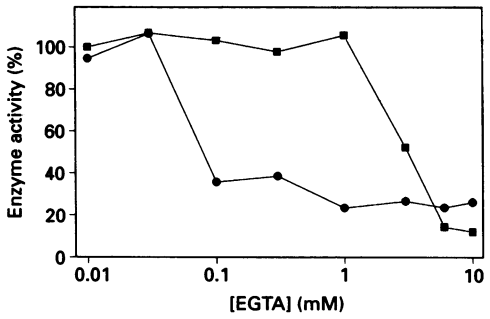

Figure 2 Effect of Ca^{2+} and EGTA on placental PLA_2

Enzyme purified by DEAE- and CM-Sephadex chromatography (150 μg per assay) was preincubated for 30 min at 0 °C with increasing concentrations of EGTA in either the absence (●) or presence (■) of 3 mM $CaCl_2$ and subsequently used in the standard assay. An activity of 100% corresponds to 6 pmol/min per sample.

Table 3 Effects of inhibitors on placental PLA_2

Enzyme purified by DEAE- and CM-Sephadex chromatography (approx. 8 pmol/min) was preincubated with the agents listed for 30 min at 0 °C in the presence of 3 mM $CaCl_2$. Assays (195 μg of protein) were started by the addition of dipalmitoylphosphatidylcholine. Experiments with aristolochic acid, CGP43182 and DTT were also carried out with stearoylarachidonyl-phosphatidylcholine and gave the same results.

Agent	Concentration	PLA_2 activity (%)
Aristolochic acid	50 μM	106
	200 μM	78
p-Bromophenacyl bromide*	500 μM	96
CGP43182	50 μM	25
	200 μM	14
Heparin	1 mg/ml	104
Quinacrine	200 μM	108
DTT	0.5 mM	69
	5 mM	16
	10 mM	6
DTT (10 mM) followed by H_2O_2	1%	119
Glutathione	10 mM	28
Iodoacetic acid	100 μM	104
Iodoacetamide	200 μM	106
2,2′-Dithiobis-(5-nitropyridine)	200 μM	102
EDTA	1 μM	30

*Preincubation was with 300 μM EGTA for *p*-bromophenacyl bromide to act [38]; 3 mM $CaCl_2$ was added to the assay.

Effects of bivalent metal ions

Enzyme assays were routinely performed in the presence of 1 mM $CaCl_2$, but no decrease in activity was observed when Ca^{2+} was omitted, indicating either a very low cation requirement or no requirement at all. When EGTA was used to chelate any endogenous Ca^{2+}, a sharp fall in activity was observed between 30 and 100 μM (Figure 2). With 3 mM $CaCl_2$ simultaneously present, the decrease occurred at 3 mM EGTA, which corresponds to 7.5 μM free Ca^{2+} as calculated by published formulae [37]. This suggests that placental PLA_2 requires roughly 10 μM free Ca^{2+} for maximum activity. Similar experiments with 3 mM $MgCl_2$ or $MnCl_2$ instead of $CaCl_2$ in the presence of increasing EGTA showed that Mg^{2+} and Mn^{2+} were not able to replace Ca^{2+} (results not shown). Interestingly, we observed a striking inhibition by Zn^{2+}, Cu^{2+} and Fe^{3+} in the absence of EGTA but

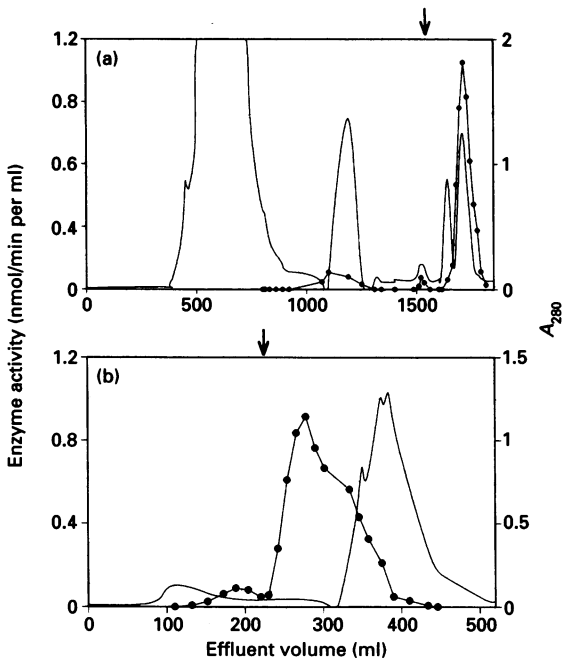

Figure 3 Purification of PLA_2 by column chromatography

(**a**) Chromatography on phenyl-Sepharose; arrow indicates start of elution with octyl glucoside. Fractions were diluted 10-fold for testing. (**b**) Chromatography on hydroxyapatite; arrow indicates start of gradient. Protein in the effluent was monitored by continuous measurement of A_{280} (——). ●, PLA_2 activity. Details are given in the Materials and methods section.

marginal effects with Mn^{2+} (results not shown). As EDTA inhibits type-II PLA_2 [29], we checked for a similar effect on the placental enzyme. Interestingly, it affected enzyme activity at a concentration as low as 1 μM (Table 3) and thus is even more potent than EGTA.

Effects of PLA_2 inhibitors

We examined the effect on the placental enzyme preparation of various inhibitors of PLA_2, in particular the $sPLA_2$ inhibitors CGP43182 [22], aristolochic acid [39,40] and heparin [21]. Table 3 shows that CGP43182 potently inhibited the placental enzyme but aristolochic acid had little effect. No inhibition was seen with heparin and quinacrine [41] nor with *p*-bromophenacyl bromide. Thiol-blocking reagents such as iodoacetic acid, iodoacetamide and 2,2′-dithio*bis*-(5-nitropyridine), all known inhibitors of $cPLA_2$ [9], did not affect placental PLA_2.

$sPLA_2$ and $cPLA_2$ forms are known to respond differently to DDT: the former characteristically contains disulphide bonds and therefore loses activity on reductive cleavage [42,43] whereas $cPLA_2$ does not [16]. Table 3 shows that placental PLA_2 is sensitive to DTT (IC_{50} 1 mM), and that glutathione similarly affected the enzyme. It is noteworthy that inhibition by disulphide reduction is completely reversed on reoxidation with H_2O_2 (Table 3), indicating that the placental PLA_2 requires intact disulphide bridges and not free thiol groups for activity.

Enzyme purification

The above properties suggest that the placental enzyme is a new form of PLA_2 distinct from $sPLA_2$ and $cPLA_2$. In particular, the approximate molecular mass fits neither group, although a dimer of 14 kDa polypeptides would produce a gel-filtration profile

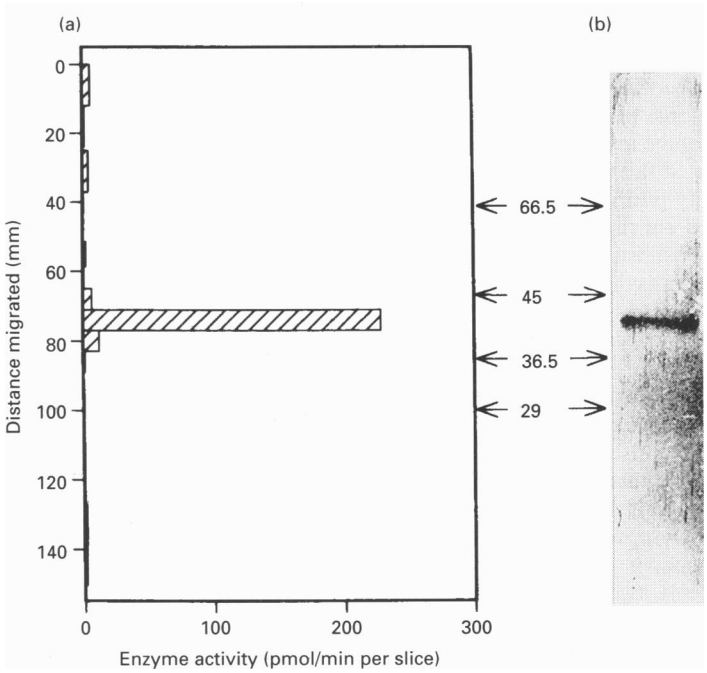

Figure 4 SDS/PAGE

(**a**) 310 μg of enzyme/lane after hydroxyapatite chromatography was subjected to SDS/PAGE under non-reducing conditions. Molecular-mass (kDa) markers are indicated by arrows. The gel was sliced as indicated, protein eluted as described in the Materials and methods section, and fractions were tested for activity (pmol/min per slice). (**b**) Silver-stained enzyme (2 μg) after gel purification and re-electrophoresis under reducing conditions.

Table 4 Reactivity of PLA_2 antisera

The recombinant enzyme was human [31] and 1 ng was used. The myeloic cells extracts were from U937 and HL-60 cells (provided by B. Yu of this Institute); 100 μg corresponding to 0.3 ng of $cPLA_2$ was used [30]. An extract from human platelets was examined; 85 μg was used roughly corresponding to 2 ng of $cPLA_2$ [46] and 0.1 ng of type-II PLA_2 [32]. The sepsis serum was 10 μg of acid-extracted human serum. Snake venom from *T. flavoviridis* (3 μg) was investigated. The placental PLA_2 (1 μg) was purified to homogeneity.

	Reactivity with antisera against:				
	$cPLA_2$			Type-II PLA_2	
PLA_2 types	Recombinant human $cPLA_2$	Residues 6–19	Residues 709–726	*T. flavoridis* venom	Residues 10–21
$cPLA_2$					
Recombinant	+	+			
Myeloic cells	+	+			
Platelets	+	+	+	−	
Type-II PLA_2					
Sepsis serum				+	+
Platelets				+	+
Snake venom			+	+	
Placental PLA_2	+	+	−	−	−

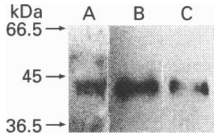

Figure 5 Immunoblot analysis

Pure enzyme (1 μg/lane) was used. Blots were stained with antisera (see Table 4) against recombinant $cPLA_2$ (lane A), the N-terminal domain (residues 6–19) of $cPLA_2$ (lane B) or a Ca^{2+}-dependent lipid-binding domain (lane C; residues 42–58 of $cPLA_2$).

similar to that of Figure 1. In order to clarify the relationship of the placental enzyme to these as well as other PLA_2s, we carried out further purification. We monitored several critical properties throughout the purification, most notably DTT sensitivity, Ca^{2+} requirement and substrate preference.

The material prepurified on DEAE- and CM-Sephadex was submitted to hydrophobic interaction chromatography on phenyl-Sepharose. After thorough rinsing at high- and low-ionic strength, the column was eluted with octyl glucoside (Figure 3a). After dialysis, the activity recovered was greater than 100% (Table 1), probably an effect of residual detergent. The enzyme was further purified on hydroxyapatite (Figure 3b). The respective purification data are listed in Table 1. As SDS/PAGE under non-reducing conditions had previously been used for the purification of several PLA_2s [44,45], we also applied this procedure (Figure 4). Gels were sliced, protein was eluted from the slices, excess SDS was removed by acetone precipitation, and individual fractions were assayed for activity. As shown in Figure 4a, PLA_2 activity was recovered in a single peak of about 42 kDa relative to the markers indicated. Preparative gel electrophoresis was repeated and yielded a preparation that gave a single band on silver staining (Figure 4b). The overall purification (Table 1) was about 15000-fold over the clear tissue extract with a recovery of 7–8%. However, the yield was 3–5-fold higher with respect to enzyme protein rather than activity. This follows from control experiments which showed that only 20–30% of activity is restored after exposure of the enzyme to SDS with subsequent removal of the detergent by acetone treatment.

Biochemical and immunochemical properties of the pure enzyme

The apparent molecular mass was determined by standard SDS/PAGE to be 42 ± 2 kDa (mean ± S.D. of three experiments) whether or not reducing conditions were used. Turnover of phosphatidylcholine with arachidonate in the 2-position is about 20-fold higher than with a saturated fatty acid at this position. This was demonstrated with highly purified enzyme using both liposomes (Table 2) and dispersed substrate (not shown). This high specificity towards arachidonyl substrate is similar to that of $cPLA_2$ [16], with substrate turnover being only slightly slower (16,30,46]. Most importantly, the effects of DDT and EGTA were maintained throughout the purification and were comparable with those of Table 3 and Figure 2 respectively.

The homogenous enzyme protein was used for immunoblotting experiments with a variety of antibodies against different PLA_2 forms (Table 4). Two independent antisera specific for type-II PLA_2 were examined. Neither of these reacted with the placental enzyme (Table 4), although materials from human sources containing this PLA_2 type (e.g. sepsis serum and platelet extract) served as positive controls. As shown in Figure 5, of three antisera specific for $cPLA_2$ (Table 4), two detected the 42 kDa placental PLA_2, albeit requiring rather high amounts of enzyme. These were prepared with the recombinant protein [31] (lane A) or a tetradecapeptide of the N-terminal region (lane B; residues 6–19). Interestingly, a peptide antibody against a C-terminal sequence (residues 709–726) of $cPLA_2$ [26] did not react (Table 4), suggesting that there was no resemblance between the placental enzyme and the C-terminal portion of $cPLA_2$. Positive reaction was also seen (Figure 5, lane C) with an antiserum against part of a characteristic Ca^{2+}-dependent lipid-binding domain (residues 42–58 of $cPLA_2$) found in various proteins [31]. We should emphasize that 1 μg amounts of the placental enzyme protein were used to produce the signals shown in Figure 5 and Table 4, whereas no more than 1 ng of protein was required in control experiments with authentic $cPLA_2$. This suggests limited cross-reactivity and explains why no immunosignals were observed when the partially purified placental PLA_2 was used (results not shown).

DISCUSSION

In the present investigation we have characterized a novel PLA_2 enzyme from human placenta which we purified to homogeneity. It has a molecular mass of about 42 kDa, as determined by SDS/PAGE, and in its native state is a monomer. This molecular mass distinguishes the placental enzyme from both $sPLA_2$s and $cPLA_2$. Nevertheless, the placental enzyme appears to share some properties with both of these enzyme classes.

Immunologically, no relationship of the placental enzyme to type-II PLA_2s was found, even though we used two independent antisera specific for this type of enzyme. The placental PLA_2 reacted weakly with an antiserum directed against a sequence close to the N-terminus of $cPLA_2$. Although the specificity of this reaction is rather low, we cannot exclude at least some distant relationship of the placental enzyme to the N-terminus of $cPLA_2$. On the other hand, there is clearly no resemblance to the C-terminal sequence of $cPLA_2$, as an antiserum specific for this region completely failed to react. Most importantly, however, the partial sequence information allows us to exclude the possibility that the placental enzyme is a proteolytic fragment of $cPLA_2$. When the N-termini of two polypeptide fragments of placental PLA_2 generated by CNBr cleavage were sequenced (11 and seven residues respectively), no similarity to $cPLA_2$ was detected (results not shown). One of these sequences contained a stretch of amino acids (ILFLLF) that is typical of proteins associated with membranes.

The fact that the placental enzyme is sensitive to DTT (Table 3) suggests the presence of disulphide bridges which are essential for activity. This property distinguishes this enzyme from $cPLA_2$ which is resistant to DTT treatment [16]. In contrast, type-II PLA_2s are sensitive to DTT [42,43]. In addition, the placental PLA_2 also shares with $sPLA_2$ enzymes a distinctive tolerance to various detergents. Of particular interest are the effects of known PLA_2 inhibitors. The active-site inhibitor CGP43182 [22] potently affects placental enzyme activity (Table 3), but the concentration required is considerably higher than that required for type-II PLA_2 inhibition [22]. Another inhibitor known to be selective for type-II enzymes [38,39], aristolochic acid, however, had little effect. This suggests that the relationship to type-II PLA_2s is also limited. Although *p*-bromophenacyl bromide blocks these enzymes by reacting with histidine in the active centre [10], it does not inhibit placental PLA_2 (Table 3). Thiol-modifying reagents (Table 2), which are known to affect various PLA_2s including $cPLA_2$ but not $sPLA_2$s [9,47,48], similarly did not inhibit the placental enzyme activity. Thus thiol groups do not appear to be involved in the active site of placental PLA_2.

Placental PLA_2 may contain a Ca^{2+}-dependent lipid-binding domain, as it reacted weakly with antiserum against part of such a sequence (Figure 5, lane C), which has been shown to be characteristically contained in proteins that act on membranes [31]. The placental enzyme requires free Ca^{2+} ions at a concentration of about 10 μM for activity. In this respect, this novel PLA_2 differs from type-II enzymes, which require millimolar concentrations of Ca^{2+} [10]. It also differs from $cPLA_2$, which, although requiring only micromolar amounts of Ca^{2+} for activity, can be further stimulated by millimolar concentrations of Ca^{2+} [16,49,50]. We assume that Ca^{2+} plays an essential role in the catalytic mechanism of placental PLA_2, but do not exclude the possibility that other metal ions may also be involved. As EDTA drastically reduced activity at concentrations as low as 1 μM (Table 3), it may chelate such ions. However, Zn^{2+}, Cu^{2+} and Fe^{3+} were found to inhibit the enzyme activity strongly. In contrast, Mg^{2+} had no effect and Mn^{2+} only little inhibitory effect (results not shown). Presumably, Mg^{2+} and Mn^{2+} do not compete with Ca^{2+} or any other bivalent cation absolutely required for activity, but Zn^{2+}, Cu^{2+} and Fe^{3+} do.

Although placental tissue was used as the source of the 42 kDa PLA_2, we wondered whether a truly cellular protein was purified. DTT sensitivity might indicate that the enzyme is secreted under certain circumstances and has an extracellular rather than an intracellular function. Indeed, in preliminary experiments we obtained evidence for a protein of roughly the same size with PLA_2 activity in serous placental fluids. Certainly, more experiments are needed to clarify the relationship between the serous fluid enzyme and the enzyme purified in this study. In particular, the cellular origin of the 42 kDa placental PLA_2 is not clear at present. It might be a gene product exclusively expressed in placental cells or it may originate from some maternal organ, perhaps the uterus. In order to address these questions, a specific antibody is required, and the generation of specific oligonucleotides would be useful for the study of expression.

It is important to compare the placental PLA_2 with canine heart and bovine brain enzymes which have similar molecular masses but are Ca^{2+}-independent [47,51]. Other properties that discriminate between the placental and myocardial enzymes are the resistance of the latter to DTT, its sensitivity to detergents and binding to an ATP affinity matrix [47].

Several groups have previously sought PLA_2 in placenta. Type-II enzyme was seen at both the mRNA [5,32,52] and

protein [6,44] levels and a type-II-related PLA_2 was detected [53]. Furthermore, the cDNA coding for a placental 30 kDa protein with PLA_2 activity has been cloned [48] and a series of 70 kDa PLA_2 isoforms have been described [45]. However, the physiological importance of any of these enzymes is not clear. To our knowledge, the presence of $cPLA_2$ in placenta has not been reported, at either the mRNA or protein level, although various cell types have been investigated [31,54]. Thus the PLA_2 species involved in physiological eicosanoid production in placenta during pregnancy and, later, at delivery is not known. Possibly, the novel 42 kDa enzyme described here serves the function of generating arachidonic acid within the placenta. The distinct preference for arachidonic acid in the 2-position of phospholipid clearly supports this view.

We are grateful to Dr. G. Fürstenberger (German Cancer Research Center, Heidelberg, Germany) and Dr. J. L. Knopf (Genetics Institute, Cambridge, MA, U.S.A.) for providing antibodies and to Dr. H. Wehrli (Ciba-Geigy, Basel, Switzerland) for the inhibitor CGP43182. Dr. J. L. Knopf and Dr. J. Aufenanger (Institute for Clinical Chemistry, Mannheim, Germany) kindly provided recombinant $cPLA_2$ and human sepsis serum, respectively. We thank B. Segnitz for help with the Figures. This work was supported by grants from the Deutsche Forschungsgemeinschaft and by Fonds der Chemischen Industrie.

REFERENCES

1 Walsh, S. W. and Parisi, V. M. (1986) Semin. Perinatol. **10**, 334–335
2 Zahradnik, H. P., Schäfer, W., Wetzka, B. and Breckwoldt, M. (1991) Eicosanoids **4**, 123–136
3 Myatt, L. (1992) Placenta **13**, 329–341
4 Challis, J. R. G. and Olson, D. M. (1988) The Physiology of Reproduction (Knobil, E. and Neill, J., eds.), pp. 2177–2216, Raven Press, New York
5 Aitken, M. A., Rice, G. and Brennecke, S. (1992) Prostaglandins **43**, 361–370
6 Andersen, S., Sjursen, W., Lægreid, A., Austgulen, R. and Johansen, B. (1994) Prostaglandins, Leukotrienes and Essential Fatty Acids **51**, 19–26
7 Pulkkinen, M. O., Kivikoski, A. I. and Nevalainen, T. J. (1993) Gynecol. Obstet. Invest. **36**, 96–101
8 Fitzgerald, D. J., Rocki, W., Murray, R., Mayo, G. and FitzGerald, G. A. (1990) Lancet **335**, 751–754
9 Mayer, R. J. and Marshall, L. A. (1993) FASEB J. **7**, 339–348
10 Kramer, R. M. (1993) Adv. Second Messenger Phosphoprotein Res. **28**, 81–89
11 Dennis, E. A. (1994) J. Biol. Chem. **269**, 13057–13060
12 Mukherjee, A. B., Miele, L. and Pattabiraman, N. (1994) Biochem. Pharmacol. **48**, 1–10
13 Burgoyne, R. D. and Morgan, A. (1990) Trends Biochem. Sci. **15**, 365–366
14 Cockcroft, S. (1992) Biochim. Biophys. Acta **111**, 135–160
15 Liscovitch, M. (1992) Trends Biochem. Sci. **17**, 393–399
16 Clark, J. D., Milona, N. and Knopf, J. L. (1990) Proc. Natl. Acad. Sci. U.S.A. **87**, 7708–7712
17 Bonventre, J. V., Gronich, J. H. and Nemenoff, R. A. (1990) J. Biol. Chem. **265**, 4934–4938
18 Xing, M., Wilkins, P. L., McConnell, B. K. and Mattera, R. (1994) J. Biol. Chem. **269**, 3117–3124
19 Riendeau, D., Guay, J., Weech, P. K. et al. (1994) J. Biol. Chem. **269**, 15619–15624
20 Bartoli, F., Lin, H.-K., Ghomashchi, F., Gelb, M. H., Jain, M. K. and Apitz-Castro, R. (1994) J. Biol. Chem. **269**, 15625–15630
21 Murakami, M., Kudo, I. and Inoue, K. (1993) J. Biol. Chem. **268**, 839–844
22 Pfeilschifter, J., Schalkwijk, C., Briner, V. A. and van den Bosch, H. (1993) J. Clin. Invest. **92**, 2516–2523
23 Lehman, J. J., Brown, K. A., Ramandham, S., Turk, J. and Gross, R. W. (1993) J. Biol. Chem. **268**, 20713–20716
24 Buhl, W.-J., Yu, B. and Gehring, U. (1995) Cell. Signal. **7**, in the press
25 Buhl, W.-J., Zipfel, M., Garcia, M. T., Eisenlohr, L. M., and Gehring, U. (1991) Eiscosanoids **4**, 235–244
26 Kast, R., Fürstenberger, G. and Marks, F. (1993) J. Biol. Chem. **268**, 16795–16802
27 Leslie, C. C. (1991) J. Biol. Chem. **266**, 11366–11371
28 Buhl, W.-J., Garcia, M. T., Zipfel, M., Schiebler, W. and Gehring, U. (1991) Eur. J. Cell Biol. **56**, 381–390
29 Ono, T., Tojo, H., Kuramitsu, S., Kagamiyama, H. and Okamoto, M. (1988) J. Biol. Chem. **263**, 5732–5738
30 Kramer, R. M., Roberts, E. F., Mannetta, J. and Putnam, J. E. (1991) J. Biol. Chem. **266**, 5268–5272
31 Clark, J. D., Lin, L.-L., Kriz, R. W. (1991) Cell **65**, 1043–1051
32 Kramer, R. M., Hession, C., Johansen, B. (1989) J. Biol. Chem. **264**, 5768–5775
33 Harlow, E. and Lane, D. (1988) Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
34 van Weemen, B. K. and Schuurs, A. H. W. M. (1971) FEBS Lett. **15**, 232–235
35 Oakley, B. R., Kirsch, D. R. and Morris, N. R. (1980) Anal. Biochem. **105**, 361–363
36 Deems, R. A. and Dennis, E. A. (1975) J. Biol. Chem. **250**, 9008–9012
37 Raaflaub, J. (1960) Methods Biochem. Anal. **3**, 301–325
38 Verheij, H. M., Volwerk, J. J., Jansen, E. H. J. M. et al. (1980) Biochemistry **19**, 743–750
39 Nakazato, Y., Simonson, M. S., Herman, W. H., Konieczkowski, M. and Sedor, J. R. (1991) J. Biol. Chem. **266**, 14119–14127
40 Rosenthal, M. D., Vishvanath, B. S. and Franson, R. C. (1989) Biochim. Biophys. Acta **1001**, 1–8
41 Mayorga, L. S., Colombo, M. I., Lennartz, M. et al. (1993) Proc. Natl. Acad. Sci. U.S.A. **90**, 10255–10259
42 Hara, S., Kudo, I., Chang, H. W., Matsuta, K., Miyamaoto, T. and Inoue, K. (1989) J. Biochem. (Tokyo) **105**, 395–399
43 Mizushima, H., Kudo, I., Horigome, K. et al. (1989) J. Biochem. (Tokyo) **105**, 520–525
44 Lai, C.-Y. and Wada, K. (1988) Biochem. Biophys. Res. Commun. **157**, 488–493
45 Jordan, L. M. and Russo-Marie, F. (1992) J. Chromatogr. **597**, 299–308
46 Kim, D. K., Suh, P. G. and Ryu, S. H. (1991) Biochem. Biophys. Res. Commun. **174**, 189–196
47 Hazen, S. L., Stuppy, R. J. and Gross, R. W. (1990) J. Biol. Chem. **265**, 10622–10630
48 Zupan, L. A., Steffens, D. L., Berry, C. A., Landt, M. and Gross, R. W. (1992) J. Biol. Chem. **267**, 8707–8710
49 Leslie, C. C., Voelker, D. R., Channon, J. Y., Wall, M. M. and Zelarney, P. T. (1988) Biochim. Biophys. Acta **963**, 476–492
50 Kim, D. K., Kudo, I. and Inoue, K. (1991) Biochim. Biophys. Acta **1083**, 80–88
51 Hirashima, Y., Farooqui, A. A., Mills, J. S. and Horrocks, L. A. (1992) J. Neurochem. **59**, 708–714
52 Crowl, R., Stoner, C., Stoller, T., Pan, Y.-C. and Conroy, R. (1990) in Biochemistry, Molecular Biology, and Physiology of Phospholipase A_2 and its Regulatory Factors (Mukherjee, A. B., ed.), pp. 173–184, Plenum Press, New York
53 Chen, J., Engle, S. J., Seilhamer, J. J. and Tischfield, J. A. (1994) J. Biol. Chem. **269**, 2365–2368
54 Sharp, J. D. and White, D. L. (1993) J. Lipid Mediat. **8**, 183–189

Received 3 May 1995; accepted 30 May 1995